Affinity capture of proteins from solution and their dissociation by contact printing

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Biological experiments at the solid/liquid interface, in general, require surfaces with a thin layer of purified molecules, which often represent precious material. Here, we have devised a method to extract proteins with high selectivity from crude biological sample solutions and place them on a surface in a functional, arbitrary pattern. This method, called affinity-contact printing (αCP), uses a structured elastomer derivatized with ligands against the target molecules. After the target molecules have been captured, they are printed from the elastomer onto a variety of surfaces. The ligand remains on the stamp for reuse. In contrast with conventional affinity chromatography, here dissociation and release of captured molecules to the substrate are achieved mechanically. We demonstrate this technique by extracting the cell adhesion molecule neuron-glia cell adhesion molecule (NgCAM) from tissue homogenates and cell culture lysates and patterning affinity-purified NgCAM on polystyrene to stimulate the attachment of neuronal cells and guide axon outgrowth.

The placement of functional molecules as biological layers on a substrate with highly controlled purity, density, functionality, and spatialization facilitates the study of biological phenomena such as protein deposition, molecular recognition, biocompatibility, and cell attachment at interfaces[1]. Conventionally, protein-coated surfaces are prepared by incubating the surface (for ~1 h) with a solution of the selected protein. This method requires concentrated solutions of purified proteins (up to 1 mg/ml), which often necessitates lengthy purification procedures[2].

We have developed a new procedure, called αCP, to extract proteins from complex media and place them as printed patterns directly on various solid substrates. The method weds the virtues of affinity purification[3] with microcontact printing (μCP) (refs 4, 5). Whereas in standard affinity-purification techniques, the elution of affinity-captured molecules is conducted by increasing the ionic strength, lowering the pH, or adding a soluble competitor, αCP releases captured molecules onto surfaces by soft contact (Fig. 1). Here, as the stamp is removed, affinity-bound target molecules dissociate and remain on the surface of the substrate in regions of contact. The capturing molecules, however, remain on the stamp and are ready for many more cycles of capturing and printing.

Stamps for αCP consist of polydimethylsiloxane (PDMS), an inert elastomer[6]. To attach the capturing molecules to the stamp in a stable manner, we used a procedure defined in previous work that includes activation of the stamp surface by an oxygen plasma, derivatization of the stamp with a silane layer and a bifunctional cross-linker, and covalent attachment of the capturing molecule through the cross-linker[7].

Results and discussion

To evaluate the performance of αCP, the transfer of ^{125}I-labeled mouse IgG to a glass surface from a stamp containing anti-mouse IgG as the capturing molecule was measured (Fig. 2). The stamp was "inked" with increasingly diluted ^{125}I-IgG in PBS containing 10% fetal calf serum (FCS), and the affinity-adsorbed protein was "stamped" onto a glass surface. For comparison, the glass surface was incubated with the same solutions of ^{125}I-IgG as used for inking to show that by this conventional adsorption technique the amount of directly adsorbed ^{125}I-IgG protein diminished rapidly because of strong competition by FCS. In contrast, the affinity-stamped radioactivity remained significant down to 1 nM ^{125}I-IgG in the presence of 10% FCS (Fig. 2A, right-hand side). In the absence of FCS, slightly less protein was transferred by αCP than by direct adsorption, probably because specific capturing has greater steric requirements than nonspecific adsorption (Fig. 2A, left-hand side). No ^{125}I-IgG was transferred from a stamp containing an unrelated capturing antibody, proving the specificity of αCP (Fig. 2A, right-hand side). After printing, <5% of the captured ^{125}I-IgG remained on the affinity stamp.

A stamp was used for 10 capturing and printing cycles to assess reusability. Protein A, a 41 kDa protein specific for the Fc part of IgG was covalently attached to the stamp, which was repeatedly incubated and printed using fluorescently tagged rabbit IgG (in PBS with 1% bovine serum albumin (BSA); Fig. 2B, dark solid line). The experiment demonstrates that protein A remains functional after repeated contact-mediated release of bound antibody. (The small loss of transfer efficiency after the first round of stamping is attributed to the release of noncovalently bound protein A from the stamp.) The release of the captured target molecules and their transfer to the substrate surface appeared to be quantitative: measuring the radioactivity and the fluorescence of the stamp after affinity printing revealed a transfer efficiency of better than 95%. Furthermore, the proteins placed on a substrate by αCP in this study and by microcontact printing in a former study[7,8] largely retained their function as verified by bioassays and immunostaining. To further generalize αCP, we used the versatile biotin-streptavidin system and printed biotinylated alkaline phosphatase as a reporter enzyme onto glass slides (Fig. 2C).

The release of captured target molecules and their transfer to the printed surface is an intriguing phenomenon and may seem counterintuitive: transfer occurs at high yield without noticeably disrupting

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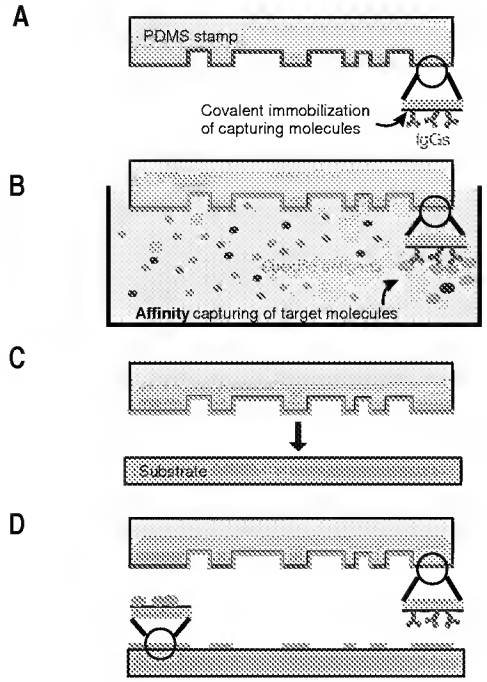

Figure 1. Affinity-contact printing extracts target molecules selectively from a medium and releases them as a printed pattern on a substrate. Affinity-contact printing involves the following steps. (A) The surface of a patterned PDMS elastomer is covalently derivatized with a layer of IgGs. (B) These IgGs selectively capture target molecules from solution. (C) After rinsing and drying, the captured molecules are transferred to a substrate in the regions of contact during a brief printing step. (D) The stamp leaves a patterned layer of target molecules on the substrate and can be reused.

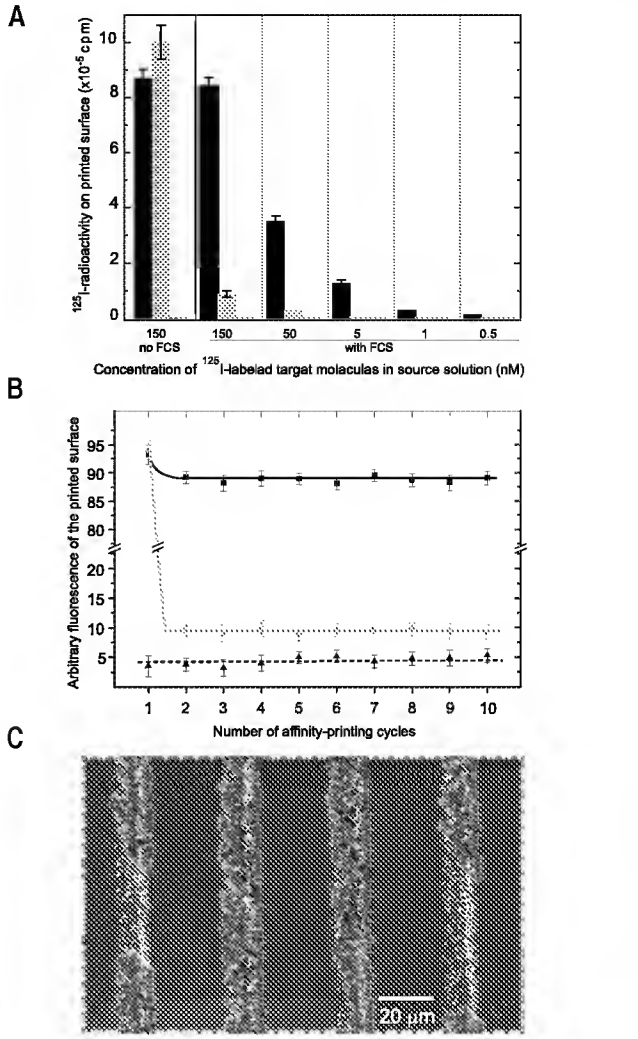

Figure 2. An αCP stamp has a high specific affinity for its target molecule and can be reused. (A) A radiolabeled antigen in the presence of 10% FCS (right) and in the absence of FCS (left) was used to determine the efficiency of αCP (left bars) in comparison to passive adsorption from solution (middle bars) and to αCP with a stamp containing a wrong capturing molecule (right bars). The amount of ^{125}I-labeled target molecules printed on glass slips is plotted against their concentration in the source solution used for inking the stamp. Using the specific radioactivity of the ^{125}I-IgG, a dense monolayer (~3 ng/mm^2) here corresponds to ~1.2×10^5 c.p.m. (B) An αCP stamp derivatized with protein A was used repeatedly to extract IgGs from a solution containing the target molecule at a concentration of 5 µg/ml in the presence of a thousandfold excess of BSA. The IgG on the glass slide was quantified by fluorescence microscopy. After each print, the same stamp was rinsed with water and re-inked with the same solution (solid dark line). Control stamps: stamp with chicken IgG as the capturing molecule (dashed line); stamp with the cross-linker quenched by incubation with ethanolamine to prevent covalent attachment of the capturing antibody (solid gray line). Fluorescence signals were corrected for background fluorescence. Error bars are calculated from three individual experiments. (C) Streptavidin immobilized as the affinity layer on a stamp served to bind biotinylated molecules from a target solution selectively and place them on a glass surface. The pattern of affinity-printed biotin-labeled alkaline phosphatase was readily visualized using the water-soluble fluorogenic substrate ELF-97, which precipitates at the site of enzymatic turnover.

the affinity layer on the stamp or destroying the printed molecules, as demonstrated by the experiments above. We can propose the following explanation based on thermodynamic and kinetic considerations. Removing the stamp from the surface after a few seconds breaks at least one of the three contacts shown in the scheme of Figure 3. Where is the weakest contact? Adhesion of proteins to a surface can be strong because proteins can cover large areas of contact (probably >100 nm^2 in the case of an IgG) and can conform to the chemical and geometric characteristics of the underlying surface, so that detachment of a protein can resist pulling forces (F_{adhes}) of >400 pN (refs 9–11). The capturing molecule is covalently linked to the stamp, and at least one bond has to be broken for detachment (F_{chem} >1 nN) (refs 10, 12). Hence, removing the stamp from the surface probably will disrupt the weakest element of the system: the specific binding interaction between the capturing antibody and the target antigen. In support of this, we note that only roughly 250 pN are necessary to disrupt the bond between biotin and streptavidin[13], a complex several orders of magnitude stronger than an antigen–antibody complex.

The unbinding forces have been shown to depend on the loading rate[14,15]; that is, the rupture force scales with the logarithm of the velocity of the pulling process. Higher forces are felt when pulling is faster. Here, it is difficult to asses the actual rupture force exerted on the molecules when the affinity stamp is lifted. Note first, however, that more or less the same pulling rate is applied to all bonds in one series, and second that the unbinding force never exceeded 500 pN even at the highest practical loading rates reported in the literature (~4,000 pN/s).

There is another argument to explain the reason why the contact between the capturing and the target molecule is the first to be broken. Binding of an antigen to an antibody is rapid ($k_{on} = 10^4 - 10^7$ M^{-1} s^{-1}). Because the dissociation constant K_d of a good antigen–antibody complex is typically of the order of 10^{-9} M, off-rate constants are 10^{-5} to 10^{-2} s^{-1} ($K_d = k_{off}/k_{on}$). These off-rates are higher than those for the desorption of a protein from a surface: target molecules are "irreversibly immobilized" and cannot be washed off easily, even by strong rinsing[7].

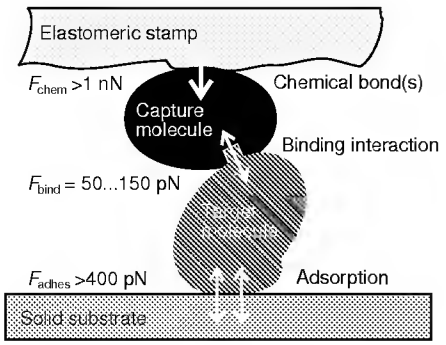

Figure 3. Scheme depicting the type and magnitude of interactions between the biomolecules and substrates during the printing step of αCP. Forces to separate two elements in this chain are estimated based on single-molecule atomic force microscopy (AFM) experiments (see text).

This seems to be the key for nondestructive release of the binding partners. The elastomeric nature of the stamp, in our opinion, merely enables the contact of two surfaces over an extended area on a molecular and atomic scale, which is crucial to the usefulness of αCP.

To what purpose might the attributes of selectivity and directed release of αCP be applied best? Neurons are particularly difficult to attach and cultivate on substrates because they require specific cell adhesion molecules with which to interact. We demonstrate the potential of αCP by patterning and immobilizing the cell growth promoting molecule NgCAM on polystyrene dishes to define sites for cell attachment and guided axon outgrowth[16]. Neuron-glia cell adhesion molecule is a 200 kDa transmembrane protein of the immunoglobulin-like superfamily. As a source for NgCAM, membrane homogenates of chicken brain containing NgCAM at a concentration of ~1 μg/ml and supernatants from COS-7 cells transfected to express NgCAM (~400 ng/ml of supernatant) were used.

Figure 4A shows the growth of dorsal root ganglia (DRG) neurons on substrates coated with conventionally purified NgCAM. Immunostaining (left) shows that NgCAM was homogeneously present on the substrate, and the amount of axonal outgrowth and fasciculation of these neurons is indicative of the suitability of these substrates (right). The concentration of NgCAM deposited on surfaces from crude solutions (natural fluids from brain or cell culture) is clearly too low to form a viable substrate for the development of DRG neurons (Fig. 4B). In contrast, culture dishes prepared by αCP with a covalently immobilized monoclonal antibody against NgCAM (monoclonal antibody E311) contained a concentration of NgCAM high enough to promote cell attachment and axon outgrowth (Fig. 4C). Cell attachment and outgrowth were almost indistinguishable from those seen on substrates coated by conventional methods (compare panels A and C). Note that no capturing antibodies were transferred together with NgCAM, as confirmed by immunofluorescence staining.

A significant feature of the αCP technique is the ease of producing patterned protein layers, which can be used to guide cell attachment and outgrowth of cells[17–21]. This remarkable possibility is demonstrated in Figure 4D. The outgrowth of axons precisely follows the striped pattern of NgCAM created with a stamp featuring a surface relief of 16-μm-wide parallel lines spaced 26 μm apart (Fig. 4D). The attachment and outgrowth of DRG neurons were suppressed by preincubating the affinity-stamped culture dish with antibody Fab fragments from a goat serum against NgCAM(ref 22). This clearly indicates that the patterned protein, which promoted axon outgrowth, is NgCAM.

Polydimethylsiloxane stamps are gas and slightly water permeable. These properties provide a benign matrix on which biomolecules are captured and held in a state preventing denaturation for a brief time (up to 1 min)[7] because buffer layers constitute a "natural" environment at the solid/liquid–air interface of the stamp. Affinity-contact printing is not limited to the use of antibodies or protein A to affinity-purify biomolecules from crude solutions. Any type of ligand–analyte interaction may be exploited. By patterning a stamp with various types of capturing molecules, "smart stamps" for screening several analytes in a parallel manner could be devised. The affinity between the capturing molecule and its target should be in the nanomolar range. Systems with lower affinity are conceivable but would require high concentrations of the target molecule, and the transfer yield may be lower. Affinity-contact printing should have general applicability not only for bioassays and cell growth experiments but also for preparing homogeneous samples for mass spectrometry and surface analysis instruments. Moreover, using "smart stamps" with arrayed capturing sites on their surfaces, protein arrays of target molecules can be fabricated in a highly parallel fashion. Thus, αCP may become a

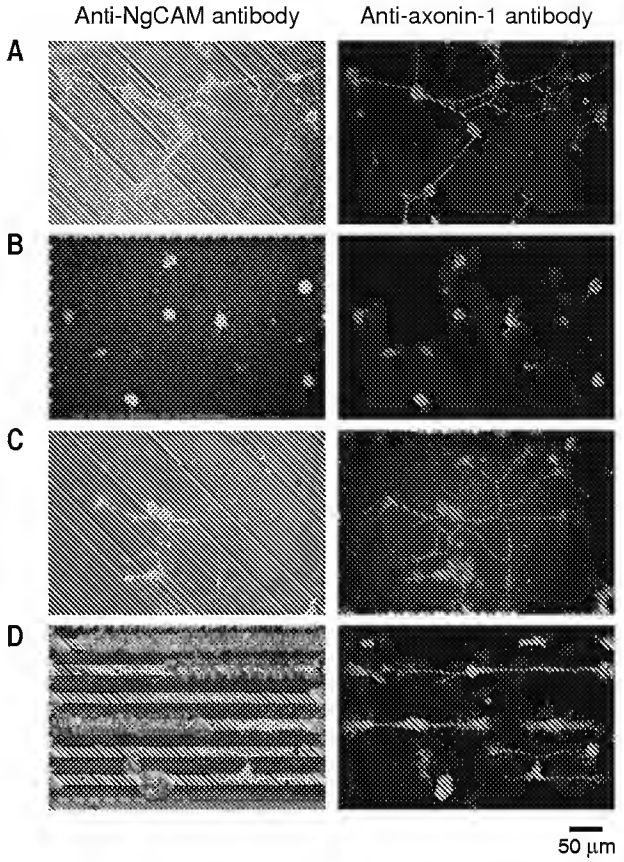

Figure 4. Attachment and development of DRG neurons coincide with NgCAM-derivatized substrates prepared either in a conventional way or using αCP. (A) Polystyrene culture dishes were coated with NgCAM (20 μg/ml) purified by standard methods and immunostained using fluorescein-labeled antibodies (left). The NgCAM serves as a good substrate for axon outgrowth of DRG cells, which were stained with rhodamine-labeled antibodies (right). (B) The source solution of brain homogenate was coated directly as is onto the cell dishes. Only background fluorescence was detected (left), and no cell growth was observed (right). This demonstrates that the amount of NgCAM present in the brain homogenate is too low to be coated unpurified and that no other components were present in a concentration sufficient to promote cell growth. (C) A flat stamp was derivatized with an antibody against NgCAM. The same solution as in (B) was used for αCP. A slightly lower fluorescence intensity than with pure NgCAM in (A) was measured, and the cell accepted affinity-stamped NgCAM as a good substrate. (D) A patterned substrate can be obtained using a stamp with a surface relief (here 16 μm lines on 26 μm spacing). Cells attach and outgrow only along the lines where NgCAM has been deposited by αCP.

versatile tool for extracting, affinity purifying, concentrating, and patterning precious biomolecules in a single step.

Experimental protocol

Preparation of stamps. Polydimethylsiloxane stamps resulted from curing Sylgard 184 (Dow Corning, Midland, MI) on fluorinated silicon masters that had etched features, 300 nm deep, on their surface. When flat unstructured stamps were needed, PDMS was cured against polystyrene dishes (Falcon, Becton Dickinson, Franklin Lakes, NJ).

Derivatization of stamps. Polydimethylsiloxane surfaces were treated in an oxygen plasma (pressure 0.8 torr, load coil power 100 W; Technics Plasma 100-E, TePla AG, Germany) for 10 s. The hydrophilic stamps were immersed immediately in an aqueous solution of 10% aminopropyltriethoxysilane (Fluka, Buchs, Switzerland) adjusted to pH of ~6.0 with acetic acid. The solution was heated for 1 h to 80°C under reflux conditions. Freshly silanized PDMS was rinsed exhaustively with deionized water (Milli-Q; Millipore, Bedford, MA). Silanized substrates can be stored up to one week when kept immersed in water. Stamps were dried under a stream of nitrogen and directly incubated with the cross-linker bis(sulfosuccinimidyl)suberate (BS3, Pierce, Rockford, IL; 1 mM in water) for 10 min. A drop (~50 µl) was applied on top of the stamp so as to cover its entire surface. After rinsing the stamp with water and blow-drying it under a stream of N_2, a drop of ~50 µg/ml of the capturing protein in acetate buffer (10 mM, pH 5.0) was applied to the reactive surface for 20 min. Stamps were rinsed thoroughly (10 ml PBS) and dried under a stream of N_2. Affinity stamps were stored in PBS buffer solution until used. The above protocol also was used under sterile conditions when appropriate. Affinity stamps derivatized with protein A (Fluka), streptavidin (Sigma, Buchs, Switzerland), anti-NgCAM monoclonal antibody 12-I-14E-311 (E311, supplied by Dr. Fritz G. Rathjen, Max Delbrück-Centrum für Molekulare Medizin, Berlin, Germany), and preimmune chicken IgG (Sigma) were prepared accordingly.

Preparation of substrates. Glass slides (Menzel-Gläser, Braunschweig, Germany) were sonicated in a 2:1 solution of water and ethanol for ~5 min and dried under a stream of N_2 before their use.

Preparation of neuronal cells and cell culture. Neurons were prepared from DRG of the E10 chick embryos, dissociated by trypsinization, and trituration as described elsewhere[23]. We purified NgCAM by immunoaffinity chromatography from detergent-solubilized brain membranes of E14 chicken cells[24]. Cells were plated at a density of 70,000 cells/10 cm^2 and cultivated for 18 h in serum-free medium as previously specified[24]. The cells were fixed by addition of formaldehyde and glutaraldehyde to a final concentration of 2% and 0.05%, respectively, and then immunostained. Functional blocking of coated or printed NgCAM was achieved by adding Fab fragments of goat anti-NgCAM serum[25].

Affinity stamping. Protein A–derivatized stamps were incubated with a solution of tetramethylrhodamine isothiocyanate (TRITC)–labeled rabbit IgG (5 µg/ml; Sigma) in PBS containing 1% BSA (Sigma) for 30 min per cycle. The stamps then were rinsed (PBS and deionized water), dried with N_2, and printed. Streptavidin-derivatized stamps were incubated with a solution of 10 µg/ml biotinylated alkaline phosphatase (Pierce) in PBS containing 1% BSA for 20 min. After rinsing, blowing dry, and stamping, the print was incubated with the fluorogenic phosphatase substrate ELF-97 (Molecular Probes, Eugene, OR) 10 µM in glycine buffer (100 mM, pH 10.0, 1 mM $MgCl_2$, 1mM $ZnCl_2$) to visualize the local enzymatic activity. Affinity stamps for NgCAM were equilibrated with a brain extract solution (from chicken, solubilized by the detergents β-octylglucoside from Sigma, and 3-[(3-cholamidopropyl)dimethylammonio]-L-propanesulfonic acid (CHAPS) from Fluka) containing ~1 µg/ml NgCAM. Lysate of transfected COS-7 cells served as another source of NgCAM. The NgCAM-loaded stamp then was rinsed with PBS (5 ml) and water (5 ml), dried in a stream of N_2, and printed onto polystyrene for 1 min. The dish and the stamp were covered with PBS before the stamp was removed to keep the printed layer under quasi-physiological conditions. Stamps with affinity for NgCAM were reused in subsequent affinity-inking/stamping cycles.

Fluorescence staining. Fluorescent images were acquired with a microscope (Labophot-2; Nikon AG, Küsnacht, Switzerland) equipped with a charge-coupled camera cooled to 0°C (ST-8; SBIG, Santa Barbara, CA), captured, and analyzed using software for this camera (SkyPro; Software Bisque, Golden, CO). Red- and green-labeled IgGs were tagged by TRITC (l_{ex} = 552 nm) and fluorescein isothiocyanate (l_{ex} = 520 nm), respectively (Sigma). We were able to image NgCAM-patterned substrates (after blocking with BSA or FCS) using a solution of goat anti-NgCAM antibody G4 (ref. 25) and subsequent immunostaining with an fluorescein isothiocyanate-labeled dog anti-goat antibody (Sigma; 1:200 in PBS). Neuronal cells were stained by rabbit anti-axonin-1 IgG (R50) and TRITC-labeled dog anti-rabbit IgG (Sigma, 1:100 in PBS).

Radioactivity measurements. ^{125}I-IgG (courtesy of L. Mastroberardino, University of Zurich, Switzerland) was used at various concentrations in PBS containing 10% FCS (Sigma). Printed glass substrates, cut into small pieces, and the stamps were rinsed with water, placed in counting vials, and measured in a γ-counter (Kontron Instruments, Zurich, Switzerland).

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